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ScienceDirect
Quaternary International 169–170 (2007) 4–16

Cyto-nuclear genomic dissociation and the African elephant species question

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Available online 16 October 2006

Abstract

Studies of skull morphology and of nuclear DNA have strongly concluded that African elephants comprise two species. Nonetheless, a recent article [Debruyne (2005). A case study of apparent conflict between molecular phylogenies: the interrelationships of African elephants. Cladistics 21, 31–50] has suggested a single-species model for *Loxodonta* based on the polyphyly of a single genetic locus, mitochondrial DNA (mtDNA). Discordant patterns between mitochondrial and nuclear DNA markers were subsequently reported in some African savanna elephant populations, further supporting a two-species model, and prompting us to re-examine here the geographic distribution of different elephant morphotypes and their relationship to nuclear and mtDNA phylogeographic patterns. We used exact tests to compare the distribution of forest elephant-typical and savanna elephant-typical characteristics across eight published datasets containing morphological, mtDNA or nuclear DNA data for African elephants. Among the elephants examined by Debruyne (2005), we found that patterns of forest vs. savanna characteristics were significantly different ($p<10^{-5}$) between mtDNA and morphology, suggesting the presence of cyto-nuclear genomic dissociation. We show that the eight African elephant continent-wide datasets compared, including that of Debruyne (2005), together support a two-species model with cyto-nuclear genomic dissociation rather than a one-species model, and together indicate that Africa harbors two species of elephant.

1. Introduction

Do African elephants comprise one or two species? All forest and savanna elephants from across Africa had been considered a single species for much of the 20th century. Recent studies have led to renewed discussion of the systematics of African elephants (genus *Loxodonta*), since both morphological and nuclear DNA data have supported their classification into two distinct species separated by a narrow hybrid zone. Skull measurements from 295 elephants of known provenance have established that forest and savanna elephants fall into two morphologically distinct groups (Groves and Grubb, 2000; Grubb et al., 2000). Nuclear DNA analyses using both slower-evolving nuclear intron sequences (Roca et al., 2001; Roca et al., 2005) and more rapidly evolving microsatellite loci (Comstock et al., 2002) have also established a deep evolutionary split between forest and savanna elephants, estimated at more than 3 million years. Only a few morphological intermediates and nuclear genetic hybrids have been detected, primarily in a zone of mixed forest-savanna habitat that surrounds the tropical forests of Africa.

Most recently, both Debruyne (2005) and Roca et al. (2005) have determined the deeper relationships present among mtDNA lineages in African elephants. Both detected two highly divergent clades, with one clade comprised exclusively of savanna elephant mtDNA haplotypes. Yet while the other clade contained all of the forest elephant mtDNA haplotypes, a considerable proportion of the haplotypes carried by savanna elephants also were in the forest elephant clade. Debruyne (2005) concluded, based on the mtDNA polyphyly, that Africa's elephants were a single species. Roca et al. (2005) found that the polyphyletic mtDNA pattern was dissimilar to patterns present among nuclear markers (cyto-nuclear genomic dissociation) in the same populations and individuals,

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doi:10.1016/j.quaint.2006.08.008

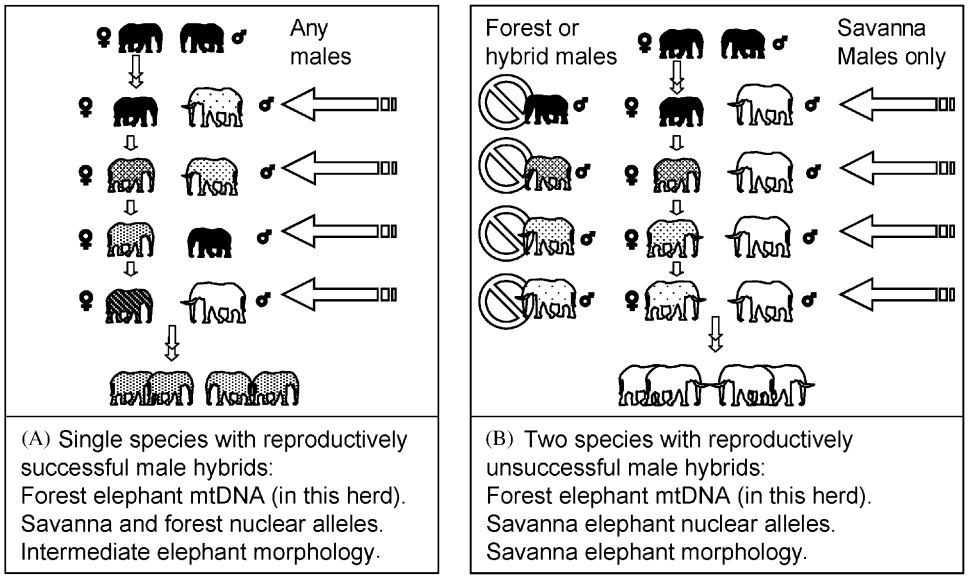


Fig. 1. Two models for the systematics of African elephants. (A) Single-species model with reproductively successful hybrids. In this model, savanna (unshaded), forest (darkly shaded) and hybrid (intermediate shades) male elephants are all reproductively successful, and there would be a close correlation among the relative number of forest versus savanna elephant mtDNA haplotypes, nuclear DNA alleles and morphological patterns in the population. The illustration does not represent every theoretical alternative in mating between forest, savanna or hybrid elephants, but is meant to suggest that forest or hybrid males and females could mate as effectively as savanna males and females under this model. (B) A two-species model suggested by the distribution of morphological types (Groves and Grubb, 2000) and based on the cyto-nuclear genomic dissociation apparent in African elephants (Roca et al., 2005). Where savanna and forest habitats meet, large savanna males gain access to forest females, enabling hybridization to occur. Given that reproductive success among male elephants depends largely on body size (Poole, 1999; Slotow et al., 2000; Sukumar, 2003), and that the deleterious effects of hybridization may differentially harm male hybrids (Haldane, 1922), recurrent backcrossing would have occurred between hybrid females and large savanna males, out-competing the smaller forest or hybrid males. The forest elephant component of the nuclear genome would be diluted and replaced in herds that retained residual maternally inherited forest-typical mtDNA haplotypes (Roca et al., 2005; Roca and O'Brien, 2005).

and therefore did not detract from the two-species model for living *Loxodonta*.

Roca et al. (2001) had argued that forest and savanna elephants comprise distinctive species based on a number of factors. These included previously established morphological and ecological differences (Grubb et al., 2000), as well as the presence of fixed nucleotide site differences, a calculated Fst between the two groups of 0.94, their phylogenetic grouping into reciprocally monophyletic clades, the large evolutionary distance between them (>3 million years), and the lack of a substantial hybrid zone or hybridization as determined by nuclear markers. Perhaps most important was the dearth of nuclear gene flow between forest and savanna populations since, at multiple loci, common alleles were restricted to only forest or only savanna populations (Roca et al., 2001).

A hybrid zone had been detected between the two species, with genotypically mixed elephants present in Garamba (Roca et al., 2001), and elephants of intermediate morphotypes present in other areas near the forest-savanna boundary (Groves and Grubb, 2000). However, the absence of intermediate morphotypes deeper in forest or savanna habitats, and the almost complete lack of detectable nuclear gene flow between forest and savanna elephants suggested that hybrids had historically been selected against. Subsequently, the lack of gene flow between forest and savanna elephant populations was inferred to be due to lack of reproductive success among hybrid males (Roca et al., 2005). Many savanna populations included a high proportion of elephants carrying forest-typical mtDNA haplotypes (Eggert et al., 2002; Debruyne, 2005). This was the case for 47 of 229 savanna individuals in the dataset of Roca et al. (2005), yet the forest elephant mtDNA proved to be "residual" since almost all of these individuals and populations lacked detectable forest elephant nuclear gene alleles.

Random hybridization under a one-species model between savanna and forest elephants could not account for the dissimilar proportions of forest-typical and savanna-typical alleles detected among different genetic markers within the same populations and individuals (Roca et al., 2005). Such intra-species hybrids would have displayed a mix of forest and savanna nuclear gene alleles (Fig. 1A). Yet such is not the observed pattern; instead, forest elephant nuclear alleles were entirely undetected in most savanna locales that display high proportions of forest-typical mtDNA. This observed pattern could only result after multiple generations of unidirectional hybridization and backcrossing of forest or hybrid females to savanna bulls (Fig. 1B) (Silver, 1995; Nagao et al., 1998; Rohwer et al., 2001; Roca et al., 2005; Roca and O'Brien, 2005). Each backcross would dilute the proportion of forest nuclear alleles by half until the populations became overwhelmingly savanna-like in nuclear genes, while retaining a maternally inherited forest-typical mtDNA haplotype (Fig. 1B) (Silver, 1995; Nagao et al., 1998;

Rohwer et al., 2001; Roca et al., 2005; Roca and O'Brien, 2005).

In many cases of hybridization between distant taxa, the deleterious effects of hybridization may differentially harm the heterogametic sex—males in the case of mammals (Haldane's rule) (Haldane, 1922; Coyne and Orr, 2004). Hybrids may be subject to extrinsic effects such as disruptions in adaptation to local environments, or intrinsic effects such as developmental defects, sterility or physiologically reduced fertility (Coyne and Orr, 2004). Among elephants, sex differences in social and reproductive behavior suggest an extrinsic mechanism (Rohwer et al., 2001; Coyne and Orr, 2004) that likely contributed to the inferred failure of forest or hybrid males to successfully reproduce in savanna locales that retain forest-typical mtDNA (Roca et al., 2005). Female elephants are philopatric (non-dispersing) and remain with their natal herd for life; males disperse at sexual maturity and mediate gene flow across herds (Poole, 1999; Nyakaana and Arctander, 1999; Sukumar, 2003). Bulls periodically enter a condition of elevated testosterone called musth, in which they pursue opportunities to mate with estrous females, and become aggressive towards competing males (Poole, 1999; Sukumar, 2003). Older larger male savanna elephants remain in musth longer than younger smaller males, and large males can suppress expression of musth in smaller males (Poole, 1999; Slotow et al., 2000; Sukumar, 2003). Since fully grown savanna bulls are almost twice as massive as forest bulls (Groves and Grubb, 2000; Grubb et al., 2000), and dominance and reproductive success are associated with larger male size (Poole, 1999; Slotow et al., 2000; Sukumar, 2003), when forest and savanna elephant males come into contact, the larger savanna males could easily out-compete forest males (Roca et al., 2005; Roca and O'Brien, 2005).

While the occasional reproductive success of a forest or hybrid male would not be precluded, the different proportions of forest-typical alleles between mtDNA and nuclear markers suggest that larger savanna bulls out-reproduced smaller hybrid males for multiple generations, leading to replacement of the nuclear genome in herds that retained the ancestral maternal forest mtDNA (Fig. 1B) (Roca et al., 2005; Roca and O'Brien, 2005). The complete lack of forest nuclear gene alleles detected among 881 Southern and 742 Eastern African savanna elephant X-chromosome segments that had been examined is indicative of overwhelming dilution of any forest elephant contribution to herd nuclear genotypes. Asymmetric hybridization appears to have produced discordant cyto-nuclear patterns in many savanna elephants, in which the mitochondrial genome is derived from a different lineage than the nuclear genes (Silver, 1995; Nagao et al., 1998; Rohwer et al., 2001; Roca et al., 2005). We would also note that the lack of savanna elephant-typical markers among African elephants in tropical forests suggests that speciation mechanisms may be expressed through other, undetermined means that prevent gene flow from savanna elephants into forest elephant population (Roca and O'Brien, 2005). Regardless of mechanism, the dearth of nuclear gene interchange between forest and savanna populations is indicative of processes that have maintained a species-level distinction between the two groups (Roca et al., 2001; Comstock et al., 2002) even in the face of historical hybridization that was geographically extensive (as inferred from the geographic distribution of forest mtDNA residual in savanna populations) (Eggert et al., 2002; Debruyne, 2005; Roca et al., 2005) and that may have increased during historical periods of habitat change. Thus although the two species hybridize, they also conform to the biological species definition of Mayr (1969) because the genetic integrity of the two parent species remains overwhelmingly intact and unmixed, even in the face of repeated historical hybridization (Roca et al., 2005).

In light of the cyto-nuclear genomic dissociation reported in many African savanna elephants (Roca et al., 2005), we here re-examine and further analyze the mtDNA and morphological dataset reported by Debruyne (2005) as well as other morphological and genetic datasets produced for African elephants. Relationships among mtDNA haplotypes, nuclear DNA genotypes and elephant morphology are examined for aspects that would be predicted to differ under a one-species versus a two-species model. We find that, for each of these aspects, the dataset of Debruyne (2005) actually supports a two species model. The morphotypes of elephants examined by Debruyne (2005) are shown to display a pattern incongruent with the pattern of their mtDNA haplotypes, suggesting that cyto-nuclear genomic dissociation is reflected in the morphology of these elephants. We also perform exact tests comparing the numbers of elephants with forest vs. savanna typical characteristics across large continent-wide datasets that have reported morphological, nuclear genetic and/or mtDNA data for African elephants. We show that published datasets, including that of Debruyne (2005), are consistent with a two-species model rather than a one-species model for Africa's elephants.

2. Methods

In our re-analysis, we relied on a number of datasets published on African elephants from a continent-wide perspective. *The morphological dataset of* Groves and Grubb (2000) includes precise morphological measurements of 295 African elephant skulls, using nine stages of tooth-eruption as a proxy for the ages of the elephants. Elephants from the forest belt of Central Africa were a priori considered to be forest elephants and those of the Eastern and Southern African savanna belt were considered to be savanna elephants. A Discriminant Analysis including measurements of crania for older elephants (50 forest and 67 savanna elephants) found differentiation between the two groups based on four morphological variables. Forest and savanna elephants separated absolutely (no overlap in Discriminant Function 1), leading the

authors to conclude that they constitute "two separate, diagnosably distinct species," with intermediate morphological types present where the ranges of the two species overlap near the boundary between tropical forest and savanna habitats.

The cyto-nuclear genetic dataset for samples collected by Georgiadis was based on DNA from soft tissue samples from 21 locations in Africa (Georgiadis et al., 1994; Roca et al., 2001). Based on appearance and habitat, elephants were classified a priori by locale as being forest elephants (3 locales), unclassified (Garamba, a region of mixed forest-savanna habitats), or savanna elephants (17 locales total: 15 designated a priori, with two unclassified Afromontane locales found to harbor only elephants with savanna elephant genotypes). These elephants have been examined with sets of largely independent markers: Four nuclear intronic sequences (three autosomal and the X-linked *BGN* gene) have demonstrated that 24 forest and 75 savanna elephants fell into two reciprocally monophyletic clades (Roca et al., 2001). A larger analysis of three X-linked intron sequences (including *BGN*) has shown almost complete separation between forest elephants (100.0% forest elephant-typical haplotypes, $n = 293$ chromosome segments) and savanna elephants (99.9% savanna elephant-typical haplotypes; with only two forest elephant-typical haplotypes among 1764 chromosome segments examined) (Roca et al., 2005). A microsatellite analysis has shown that forest and savanna elephants have species-specific genotypes (100% of 147 savanna elephants could be correctly assigned based on genotype; while all forest elephants were correctly assigned, exclusive of hybrids in Garamba) (Comstock et al., 2002). Y-chromosome intronic sequences have shown the same pattern, with deep separation between forest elephant haplotypes (30 of 30 fell within a forest-typical clade) and savanna elephant haplotypes (175 of 176 in a savanna-typical clade, with only one exceptional hybrid) (Roca et al., 2005). Yet the mtDNA of elephants in these locales was found to have a discordant pattern, with 47 of 229 savanna elephants carrying a mitochondrial haplotype from the forest elephant clade (though no forest elephant carried a savanna elephant-typical haplotype) (Roca et al., 2005). Thus cyto-nuclear genomic dissociation is present among many savanna elephant populations.

The mtDNA and morphological dataset of Debruyne (2005) was derived from African elephant museum specimens of established provenance examined for morphological features and mtDNA haplotypes, although nuclear loci were not genotyped. A larger dataset mentioned within the study was not considered here because information on the provenance of the elephants was not reported. Among elephants in the smaller dataset for which provenance was reported, 28 displayed savanna elephant morphology. Of the 28 savanna elephants identified by morphology, five carried mtDNA haplotypes typical of forest elephants while 23 carried savanna-elephant typical mtDNA haplotypes. Two elephants with intermediate morphologies were detected, from Katanga and Kanyatsi in the Democratic Republic of Congo. All elephants with forest or intermediate morphotypes carried forest clade mtDNA.

Other datasets: Other important studies reporting continent-wide mitochondrial datasets have been published (Eggert et al., 2002; Nyakaana et al., 2002). We focus on the mtDNA results of Debruyne (2005) and Roca et al. (2005) as representative of mtDNA studies because the split of African elephant mtDNA into two very divergent clades is evident with high bootstrap support in both of these datasets. By contrast, the sequences of Nyakaana et al. (2002) and Eggert et al. (2002), as combined and published by Eggert et al. (2002), had weak statistical support for many of the deeper relationships between clades. This is probably due to a combination of short sequences, which preclude strong statistical support, and to the use of the control region, which is subject to saturation and does not conform to a molecular clock (Ingman et al., 2000). Since the deeper relationship among haplogroups is unclear in Eggert et al. (2002), the dataset cannot be directly compared to subsequent mtDNA datasets. However, we would note that the relationships *within* haplogroups, in terms of inclusion of forest and/or savanna elephants and in terms of phylogeographic distribution, are nonetheless consistent with the Debruyne (2005) and Roca et al. (2005) datasets.

Nuclear DNA sequences of mitochondrial origin (numts). We believe that numts would not be responsible for the mtDNA patterns across elephant DNA studies for a number of reasons. First, technical steps were taken to minimize the possibility of amplifying numts. In Roca et al. (2005), multiple pairs of primers were used that amplified overlapping sequences, which were identical in sequence in the region of overlap, minimizing the possibility that primer mismatches had led to selective amplification of numts over cytoplasmic mtDNA. Primers were also designed for regions conserved either across large numbers of elephants of all living species or between elephants and aardvark, in order to minimize mismatches in the target DNA versus the primers. Amplicons were as large as 2.5 kb, which would avoid those numts that were shorter. Obvious indications of numts were not present, e.g., PCR did not produce multiple bands, nor were sequences heteroplasmic or show secondary peaks, while open reading frames within coding regions were conserved. Furthermore, although the different mtDNA studies used different primers to amplify various regions of the mtDNA, similar phylogeographic clusters of elephant mtDNA haplogroups were consistently generated across studies. Studies that used sequence sufficient to generate high statistical support among deeper nodes reported similar phylogenetic patterns even when using different mtDNA regions and primers. The time between forest and savanna elephant divergence (>3 mya) would likely have resulted in sequence differences between mtDNA and any numt integrations that predated this split, yet the sequences found in forest and savanna elephants were often identical, suggesting

relatively little evolutionary distance between them. Most importantly, numts should behave as nuclear markers and follow patterns detected among other nuclear markers. Nuclear markers, whether slow or fast evolving, showed relatively deep separation between savanna and forest populations, much more modest separation between Cameroon and Southern/Eastern elephants, and very little differentiation between or within south and east Africa. The mtDNA patterns, if due to numts, would be expected to follow this pattern, but are quite different from it. Indeed, given that numts would be transmitted across the savannas by male elephants, it is difficult if not impossible to imagine that a distinctive numt would be found in 90% of savanna elephants in Serengeti, but 0% of the savanna elephants in nearby Kenya, when there is no other evident nuclear genetic differentiation between the two locales, even using the fastest evolving nuclear markers. Since the mtDNA sequences are indicative of millions of years of separation, it would be difficult to see how a nuclear marker transmitted by males would remain present at one savanna locale while remaining absent from a nearby savanna locale in which the elephants are genetically indistinguishable by microsatellites and in which the locales would be linked by male-mediated gene flow. The geographic pattern of the mtDNA sequences is thus consistent with a marker transmitted by the philopatric females, but inconsistent versus that seen for any nuclear markers, whether slow- or fast-evolving, since the nuclear markers are subject to male-mediated gene flow.

Exact tests comparing two datasets for the number of forest-typical and savanna-typical characteristics among elephants in savanna habitats were performed in Arlequin version 2001 (Schneider et al., 2001), with 100,000 steps in Markov chain and 4000 dememorization steps. In cases where the exact test could not be run because both datasets had a value of zero for forest-typical characteristics, we obtained an approximate result by adding a value of one to both of the categories, forest-typical and savanna-typical, in both datasets.

3. Results

We examined four aspects of the relationships expected among the morphology of African elephants, their nuclear DNA genotypes and their mitochondrial DNA haplotypes (Table 1). The relationships predicted among these would differ under the two-species model for African elephants as elucidated by Roca et al. (2005), versus the one-species model proposed by Debruyne (2005) (Table 1). One of the major expected differences between a single-species model for African elephants and a two-species model is the degree to which the *morphology of elephants would be predicted to*

Table 1
The Debruyne (2005) dataset of morphological and mtDNA markers supports a two-species model

Attributes	Prediction under model of Roca et al (2005), *two species* with reproductively unsuccessful hybrids	Prediction under model of Debruyne (2005), *one species* with reproductively successful hybrids	Pattern present in the Debruyne (2005) morphological and mtDNA dataset	Pattern in Debruyne (2005) dataset favors:
Correlation in savanna locales between proportion of forest elephant mtDNA and elephant morphotypes	No correlation in savanna locales; forest mtDNA is relictual so a forest elephant contribution would not be reflected in nuclear DNA or morphology	Should be highly correlated since mtDNA reflects overall population structure. Forest-like or intermediate nuclear DNA and morphology should predominate where forest elephant mtDNA is high	No correlation in savanna locales. Forest and intermediate elephant morphologies are not evident in savanna populations.	Two-species model
Elephant morphology in savannas where forest elephant mtDNA predominates	Savanna elephant morphology (forest elephant mtDNA in savannas is not reflective of nuclear DNA or morphology following generations of reproductively unsuccesful male hybrids)	Forest elephant morphology and intermediate morphologies should be common since the mtDNA is reflective of successful hybridization	Savanna elephant morphology only	Two-species model
Continental distribution of morphological types	Forest elephant morphology in tropical forests; savanna elephant morphology in other habitats; limited distribution of locales with intermediate morphologies	Intermediate morphologies common across forest and savanna habitats	Forest elephant morphology in tropical forests; savanna elephant morphology in other habitats; limited distribution of locales with intermediate morphologies	Two-species model
Distribution of elephants with intermediate morphologies	Limited to habitat contact zones (or regions of anthropogenic disruption)	Common across the savannas, wherever forest and savanna elephant mtDNA haplotypes are both present	Limited to habitat contact zones	Two-species model

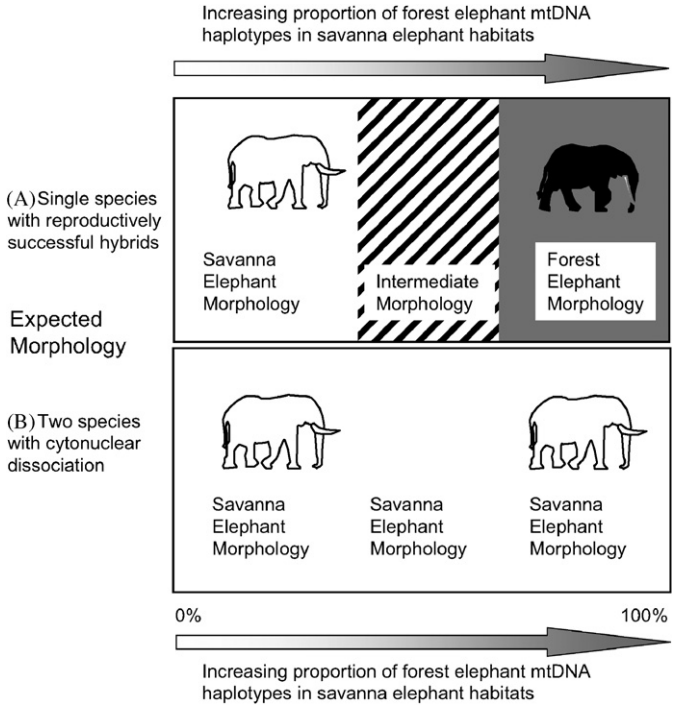


Fig. 2. The correlation between mtDNA haplotypes and morphology in a population of elephants would be expected to differ between two hypotheses: (A) a single-species model with reproductively successful hybrids (Fig. 1A), where forest mtDNA haplotypes are mirrored by extensive introgression of forest elephant nuclear alleles into savanna locales, resulting in intermediate or forest morphotypes; or (B) a two-species model where repeated unidirectional hybridization (Fig. 1B) has led to cyto-nuclear genomic dissociation in herds which carry forest elephant mitochondrial genomes but overwhelmingly savanna elephant nuclear gene alleles, leading to savanna morphotypes uncorrelated with the proportion of forest elephant mtDNA in the population.

correlate with mitochondrial haplotype (Table 1), i.e., the degree to which elephants living in savannas that carry forest elephant-typical mtDNA haplotypes would be expected to also carry forest elephant-typical nuclear gene alleles that in turn would affect morphological features (Figs. 1 and 2). Under a single-species model in which hybrid elephants are reproductively successful (Fig. 1A), populations in savanna locales that carry forest elephant-typical mtDNA would also be expected to carry a proportionate number of forest elephant nuclear alleles, and thus to exhibit forest elephant-typical or intermediate morphology (Figs. 1A and 2A). This is an assumption made by Debruyne (2005) in his assertion that "interbreeding has had a major impact on the reciprocal integrity of extant forest and savanna elephants." Yet despite this assertion, elephants with forest or intermediate morphotypes are absent from regions of savanna vegetation in the morphological datasets both of Debruyne (2005) and of Groves and Grubb (2000). This observation instead would support a two-species model where male hybrids are reproductively unsuccessful, and in which elephants from savanna locales carrying forest-typical mtDNA would nonetheless be expected to display savanna elephant morphology since they lack the nuclear alleles necessary for forest elephant or intermediate morphology (Figs. 1B and 2B).

This conclusion is especially evident when one considers *elephant morphology in savanna regions where a majority of elephants carry forest clade mtDNA haplotypes* (Table 1). Forest elephant mtDNA predominates in parts of northern Tanzania, Zimbabwe and Botswana, with up to 90% of savanna elephants (in Serengeti) carrying forest clade mtDNA (Roca et al., 2005). Under a single species model with reproductively successful hybrids, the proportion of forest-typical mtDNA in a population should be mirrored by the overall nuclear genotype and thus these regions should have predominantly forest elephant nuclear alleles and therefore forest elephant and intermediate morphotypes (Figs. 1A and 2A). Yet, in the geographically extensive and numerically large morphological datasets of both Debruyne (2005) and Groves and Grubb (2000), not a single elephant in these regions is recognized as having a forest elephant or even intermediate morphotype; all specimens from these regions are identified as savanna elephants based on morphology, even those identified by Debruyne (2005) as carrying forest mtDNA. Thus although Debruyne (2005) asserts that "interbreeding has had a major impact on the reciprocal integrity of extant forest and savanna elephants," this is not corroborated by the morphology of elephants from savanna regions where forest elephant mtDNA predominates, as would be expected under a single species model (Figs. 1A and 2A). The larger the proportion of elephants in savannas found to carry forest-elephant mtDNA haplotypes, the more surprising is the lack of individuals with forest elephant or intermediate morphology in these African savannas, and the greater the support for a two-species model in which forest elephant mtDNA is residual in savanna elephants and not reflective of their nuclear alleles or morphology (Figs. 1B and 2B).

The expected *geographic distribution of elephants with savanna-typical and forest-typical morphology* would be predicted (Table 1) to differ under a one-species versus a two-species model. Fig. 3 maps the distribution of different habitats in Africa. Under a single species model with reproductively successful hybrids (Fig. 1A), regions with forest-typical mtDNA haplotypes (ovals on the map in Fig. 3) contain hybrid elephants that should also carry forest elephant nuclear alleles and thus should have forest elephant or intermediate morphology (Figs. 1A and 2A). These regions include much of East and South-Central Africa. Yet these large swathes of territory do not contain a single elephant identified in the Debruyne (2005) dataset as having forest elephant morphology, or even intermediate morphology. Nor are elephants with forest elephant or intermediate morphologies present in these regions in the larger dataset of Groves and Grubb (2000). It is especially surprising, given the criticism by Debruyne (2005) of the use of habitat as a surrogate for the assignment of elephants, and given his determination to examine the "morphotype of the specimens that were sequenced," that

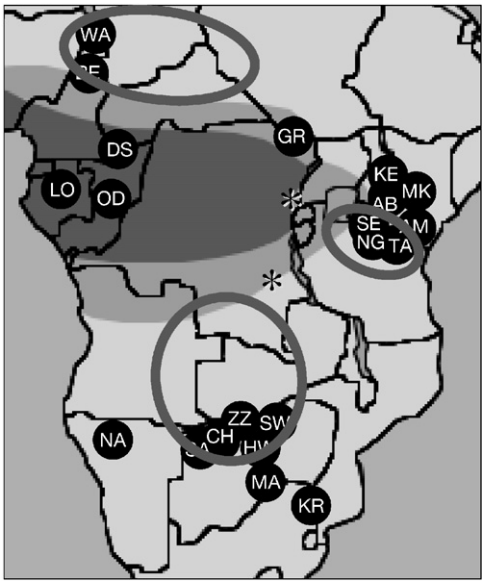


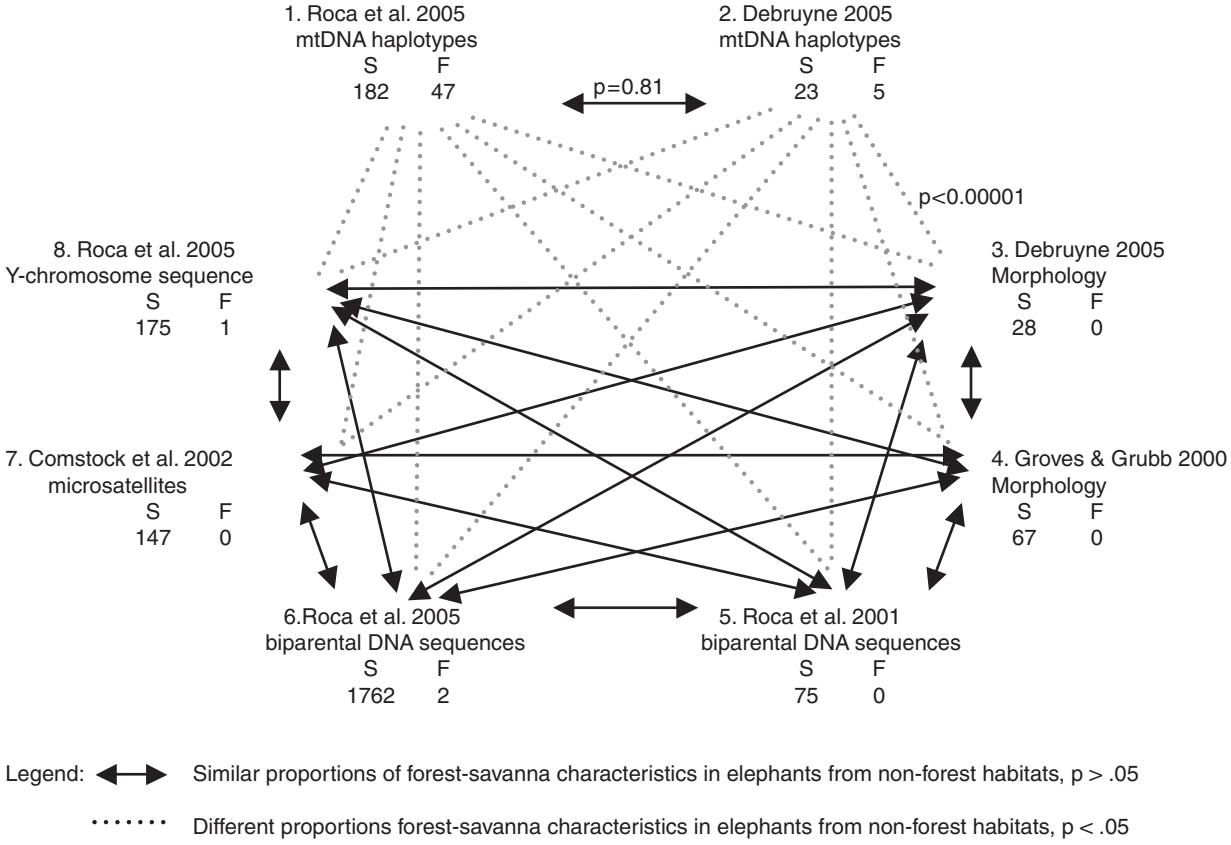
Fig. 3. Map of Africa showing historic distribution of tropical forest habitats (dark shading), forest-savanna mosaic habitats (intermediate shading) and elephant range outside of tropical forests (light shading) (White, 1983). Dark ovals indicate savanna regions where forest elephant-typical mtDNA haplotypes are common in the datasets of Debruyne (2005) and Roca et al. (2005). Asterisks indicate the approximate provenance of two elephant specimens found to have intermediate morphology by Debruyne (2005). The locations of samples collected by Georgiadis are indicated (Georgiadis et al., 1994; Roca et al., 2001). Forest locations: DS-Dzanga Sangha in Central African Republic; LO-Lope in Gabon; OD-Odzala in Congo (Brazzaville). Savanna locations: BE-Benoue and WA-Waza in Cameroon; AB-Aberdares, AM-Amboseli, KE-Central Kenya and MK-Mt. Kenya in Kenya; SE-Serengeti, NG-Ngorongoro and TA-Tarangire in Tanzania; HW-Hwange, SE-Sengwa and ZZ-Zambezi in Zimbabwe; KR-Kruger in South Africa; CH-Chobe, MA-Mashatu and SA-Savuti in Botswana; NA-Namibia. The location GR-Garamba in Congo (Kinshasa) is in the Guinea-Congolian/Sudanian transition zone of vegetation (White, 1983) that includes a mixture of forest and secondary grasslands.

this complete lack of morphological support on a continent-wide level for a single-species model does not receive greater mention. The distribution of forest elephant morphology across Africa (Groves and Grubb, 2000; Debruyne, 2005) does not follow the geographic distribution of forest mtDNA clades that extend into savannas (Eggert et al., 2002; Debruyne, 2005; Roca et al., 2005), contradicting the single-species model. However, the morphotypes of the specimens examined by Debruyne (2005) are completely consistent with the morphological dataset of Groves and Grubb (2000) and with the nuclear gene phylogeographic patterns detected by Roca et al. (2001, 2005) and Comstock et al. (2002), all of whom proposed a two-species model. Under the two-species model of Roca et al. (2005), forest typical mtDNA would be residual in elephants in savanna habitats, since unidirectional backcrossing to savanna elephant males (Fig. 1B) would have replaced the forest elephant nuclear genetic contribution, explaining the absence of forest elephant or intermediate morphotypes from savanna regions (Fig. 2B).

The actual distribution of elephants with intermediate morphologies (Table 1) includes locales in or near the region where forest and savanna habitats meet (Fig. 3), and is consistent with a two-species model as proposed by Groves and Grubb (2000) on morphological grounds and by Roca et al. (2001, 2005) on the basis of nuclear DNA. Under this model, hybrid offspring may be continuously generated where the two species presently meet. However, since the male hybrids are evolutionary "dead ends" with relatively low reproductive success (inferred from the discrepant patterns between mtDNA and nuclear markers), the geographic distribution of elephants of intermediate morphologies or of mixed nuclear genotypes is circumscribed. Although Debruyne (2005) argues that the presence of elephants with intermediate morphology in his dataset contradicts the two-species model, he lists only two of intermediate morphology among his elephants with reported provenance. One of these is from Katanga, a large province in the Democratic Republic of Congo where tropical forest meets the surrounding savanna habitats. The other intermediate morphotype is from Kanyatsi in northeast D. R. Congo, a region of tropical forest also adjacent to the intermediate zone of vegetation. Thus the provenance of both elephants of intermediate morphology (asterisks on map in Fig. 3) reported by Debruyne (2005) conforms to the geographic distribution expected under a two-species model. If one is to consider the assertion of Debruyne (2005) that "the hybrid zone between *africana* and *cyclotis* is not fairly 'narrow'," then one must note that elephants of intermediate morphology in his dataset and that of Groves and Grubb (2000) are largely limited to the fairly narrow current region where forest and savanna habitats overlap. This distribution is only consistent with a two-species model with cyto-nuclear genomic dissociation, where the fairly large geographic extent of "residual" forest clade mtDNA is due to unidirectional hybridization along a historically shifting hybrid zone between species (Rohwer et al., 2001; Roca and O'Brien, 2005), and not reflective of current morphology or nuclear genotypes.

Exact tests (Schneider et al., 2001) were used to contrast the number of forest and savanna elephant characteristics detected by different continent-wide studies that used morphological, nuclear DNA or mtDNA approaches (Fig. 4). In each case, the dataset was treated as categorical data with either "forest-typical" or "savanna-typical" characteristics, as described in the Fig. 4 legend. The distribution of forest and savanna elephant characteristics among elephants in savanna locales was compared between pairs of datasets. The datasets fell into two groups (Fig. 4A). All comparisons involving a dataset comprised of nuclear DNA or morphological traits versus another dataset comprised of nuclear DNA or morphological traits showed no significant differences in the proportion of savanna- and forest-typical characteristics between the two datasets. This indicates that all continent-wide datasets of morphology or of any type of nuclear DNA markers show similarly low numbers for forest elephant-typical traits

(A) Exact tests comparing forest and savanna elephant characteristics reported in savanna populations by different studies



(B) Exact test for difference in frequencies in forest- and savanna-typical characteristics in savanna populations, p values
Datasets used in pairwise comparisons numbered as above

	1	2	3	4	5	6	7
2	**0.81**						
3	<0.00001*	**<0.00001***					
4	<0.00001*	<0.00001*	>0.05†				
5	<0.00001*	<0.00001*	>0.05†	>0.05†			
6	<0.00001*	<0.00001*	1.00	1.00	1.00		
7	<0.00001*	<0.00001*	>0.05†	>0.05†	>0.05†	1.00	
8	<0.00001*	<0.00001*	1.00	1.00	1.00	0.25	1.00

* significant difference between two datasets in frequency of forest-typical characteristics in savanna population
† Direct test not possible since both datasets had zero forest-typical characteristics, but p>0.05 in modified test (see Methods)

Fig. 4. Exact tests comparing forest elephant-typical versus savanna elephant-typical characteristics in eight continent-wide datasets of African elephants. Only elephants from locations outside of tropical forest or intermediate habitats were considered for each dataset. (A) Exact tests between pairs of datasets indicated significant differences in the number of savanna vs. forest typical characteristics where the datasets are connected by a dotted line. By contrast, where two datasets had similar distributions of savanna vs. forest typical characteristics, they are connected by a double arrow. Datasets are identified by author, publication date, type of data, and the number of elephants in savanna locales having either savanna elephant-typical ("S") or forest elephant-typical ("F") characteristics, defined, respectively, for the numbered datasets as follows: (1) Clade I vs. Clade II mtDNA (Roca et al., 2005); (2) Clade S vs. Clade F mtDNA (Debruyne, 2005); (3) "morphological type" assignment to *africana* or *cyclotis* form (no intermediates were present in savannas) (Debruyne, 2005); (4) separation by several morphological traits (Discriminant Function 1) into forest and savanna morphological groups (no intermediates were present in savannas) (Groves and Grubb, 2000); (5) savanna and forest elephant clades inferred using four nuclear gene sequences (Roca et al., 2001); (6) savanna and forest elephant-typical haplotypes (with fixed differences) for three X-linked genes (one gene overlaps with dataset 5) (Roca et al., 2005); (7) assignment by genotypes of 14 microsatellites as savanna or forest elephants (Comstock et al., 2002); (8) Clade I vs. Clade II using a Y-chromosome sequence (Roca et al., 2005). (B) Exact test *p*-values between datasets; asterisks indicate significant differences between datasets, which are numbered as in the top panel.

among elephants in savanna locales. This pattern holds true even for the morphological dataset generated by Debruyne (2005), for which the distribution of morpho-types in savanna locales (no specimens with intermediate- or forest-elephant morphology in the savannas) was similar to the morphological pattern reported by Groves and

Grubb (2000), and also similar to each of the nuclear DNA datasets, which detected few or no forest genotypes in savanna locales (Roca et al., 2001; Comstock et al., 2002; Roca et al., 2005).

While all nuclear DNA and morphological datasets unambiguously displayed a lack of forest elephant characteristics for populations in savannas (Fig. 4), both of the mtDNA datasets demonstrated significantly higher levels of forest elephant typical characteristics in savanna locales than any nuclear DNA or morphological dataset ($p<10^{-5}$ in all comparisons). Furthermore, the only dataset with a high level of forest elephant-typical characteristics within savanna locales like that reported by Debruyne (2005) for mtDNA was the mtDNA dataset for the savanna elephants typed by Roca et al. (2005) (Fig. 4). Thus, contrary to the assertion of Debruyne (2005), the genetic composition of the Roca et al. (2001, 2005) elephants does not "conflict" with that of the Debruyne (2005) elephants, at least for the only genetic locus examined by the Debruyne (2005) study. Additionally, a lack of association between mtDNA and morphology is evident even among Debruyne's (2005) elephants, where the presence of forest elephant mtDNA in savanna locales is not associated ($p<10^{-5}$) with forest elephant or intermediate morphology. The discordance between mtDNA and morphological patterns in Debruyne's (2005) savanna elephants likely reflects the discordance between mtDNA and nuclear markers detected in other savanna elephants by Roca et al. (2005), and lends support to the two-species model inferred from the latter (Roca et al., 2001; Comstock et al., 2002; Roca et al., 2005; Roca and O'Brien, 2005).

4. Discussion

We considered several interrelated aspects of the relationships among mtDNA haplotypes, nuclear gene alleles and morphology (Table 1) that would be predicted to differ under two models proposed for African elephant systematics: a single-species model with reproductively successful hybrids (Debruyne, 2005); and a two-species model in which the relative lack of reproductive success among hybrid males keeps the two species intact but leads to polyphyly for a single genetic locus (mtDNA) (Roca et al., 2005). Each aspect considered here supported the two-species model over the one-species model.

We also determined that the ratio of forest-clade to savanna-clade haplotypes in savanna elephants is similar ($p=0.81$; Fig. 4) between the mtDNA datasets of Debruyne (2005) (5-F:23-S) and that of Roca et al. (2005) (47-F:182-S). Thus, there is no "apparent conflict" between the two datasets, and several ad hoc hypotheses proposed to reconcile the "conflict" (Debruyne, 2005) between the datasets would be moot: One hypothesis was the supposed lack of sampling locales in the Roca et al. (2001) dataset. In fact, there was extensive overlap of sampling locales between that dataset and the samples of Debruyne (2005), since both included elephants from, e.g., Tanzania and Zimbabwe. Furthermore, the "synoptic display" generated by Debruyne (2005) to represent the results of genetic datasets oversimplifies sampling in the Roca et al. (2001) dataset by labeling its elephants to be "south-eastern Loxodonta." While the "south-eastern" designation might be useful to the hypothesis of isolation by distance proposed by Debruyne (2005), the elephants of Roca et al. (2001) included many samples quite distant from south-eastern Africa, notably those from Cameroon, and these grouped definitively with other savanna elephants. Thus elephants from Cameroon were similar in nuclear DNA sequence to elephants from Namibia, and relatively dissimilar to elephants from geographically much closer forest locales. Additionally, a Mantel test had been conducted which ruled out isolation by distance as responsible for the pattern detected with nuclear genes (see Roca et al., 2001). Therefore the assertion by Debruyne (2005) that the nuclear DNA results of Roca et al. (2001) were based on "the geographic structuring of the molecular diversity rather than on the taxonomic differentiation of African elephants" had already been disproven (see Roca et al., 2001). There is also no need to posit that "sampling differences" account for the "non-representativeness of nuclear results" (Debruyne, 2005) since the nuclear results of Roca et al. (2001, 2005) are representative of populations with a proportion of forest elephant clade mtDNA similar to that of the Debruyne (2005) dataset, while the latter includes no nuclear DNA results that can be compared. Likewise, we reject the assertion by Debruyne (2005) that elephants in the Roca et al. (2001) dataset were designated as forest or savanna populations after genotyping, as almost all populations were assigned a priori (see Roca et al., 2001). And despite Debruyne's (2005) criticism of a lack of quantitative morphological measurements as the basis for species assignment, for most of these locales the morphological criteria used are the same ones used by Debruyne (2005): "for living elephants this characterization was made using classical features: height, form of the ears and the head, shape and set of the tusks, and curvature of the spine."

On closer examination, the arguments for the single-species model based on mtDNA polyphyly appear to be based on two errors in the interpretation of genetic analyses: attributing differences in tree topology across genetic markers to differences in evolutionary rates; and assuming that an unrepresentative polyphyletic gene tree can represent a species tree.

Different phylogenetic or phylogeographic patterns between nuclear and mtDNA markers cannot be attributed to their different evolutionary rates. In the attempt to reconcile the different topologies of trees inferred from nuclear DNA markers (reciprocal monophyly between forest and savanna elephants) versus trees inferred from mtDNA (polyphyletic), one must not equate differences in phylogenetic or phylogeographic patterns with differences in marker evolutionary rates. Debruyne (2005) states that his analysis would "focus exclusively on" mtDNA because "its high

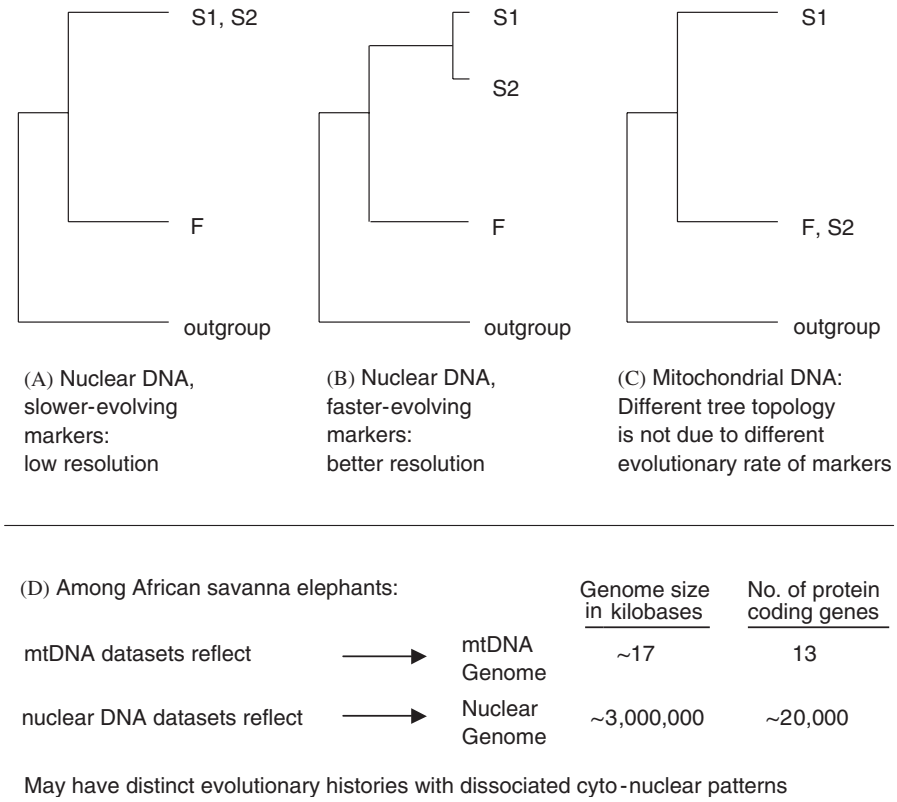


Fig. 5. (A–C) Differences in evolutionary rates among markers in elephants cannot account for trees with very different topologies. A fast evolving nuclear marker (B) may resolve differences between S1 and S2 that a slower evolving marker (A) could not resolve. However, very different tree topologies (A vs. C) between markers cannot be accounted for by differences in their evolutionary rates. (D) Due to cyto-nuclear genomic dissociation (Fig. 1B), the mtDNA tree in savanna elephants may reflect the genetic structure of a single locus, the mtDNA genome, and be quite different from the pattern demonstrated by markers in the nuclear genome (Roca and O'Brien, 2005).

level of variability suggested that it might reveal more topological resolution than nuclear markers.'' Yet we would note that the undeniably faster evolutionary rate of the mtDNA genome is completely irrelevant to the observation of distinctive patterns detected by nuclear vs. cytoplasmic markers. This is illustrated in Fig. 5, which shows how markers with different evolutionary rates would vary in their expected resolution of differences between two savanna elephant lineages. While a faster evolving genetic marker can certainly resolve more recent evolutionary events (Fig. 5B vs. 5A), differences in evolutionary rates between markers would not account for completely different tree topologies among markers (Fig. 5C vs. 5A). Debruyne (2005) notes that ''we assume that, even if built on 195 [elephant] specimens, the nuclear affinities are underestimated relative to the mitochondrial ones,'' yet this again confuses evolutionary rate differences with fundamental differences in cyto-nuclear tree topologies. The empirical data also contradicts this assertion by Debruyne (2005). When nuclear markers that evolve rapidly, such as microsatellites, have been examined, the deep and almost complete genetic separation between forest and savanna elephant populations has been strongly confirmed (Comstock et al., 2002). Although the microsatellites resolved more subtle evolutionary differences within savanna elephants, the overall pattern was completely consistent with that of slower-evolving nuclear markers, but discordant versus the mtDNA pattern.

A gene trees is not a species tree. Debruyne (2005) also argues that mtDNA is ideal for ''getting rid of tokogenetic [intra-lineage] effects in infra-specific analyses for such a matriarchal taxon,'' yet it is precisely these male-mediated tokogenetic effects which produce cyto-nuclear genomic dissociation and maintain the species barrier between savanna and forest elephants, but which are also completely indiscernible by mtDNA analyses. The mitochondrial genome is the only genetic locus that cannot be transmitted by males. Since elephant females are philopatric and live in matrilineal herds (Sukumar, 2003), mtDNA reflects the origins of the herd but may be unreflective of the extensive historical gene flow between herds mediated by males. Males carry between herds both biparentally transmitted (diploid autosomal and haplo-diploid X-chromosome) and male-transmitted (Y-chromosome) loci. All sets of nuclear genetic markers that have been examined, including nuclear gene sequences, autosomal microsatellites, X-linked gene sequences and Y-chromosome sequences, have indicated without exception that forest- and savanna- elephants form two very distinctive groups, with little hybridization or overlap of haplotypes between them (Roca et al., 2001; Comstock et al., 2002; Roca et al., 2005). While the same individuals and

populations may demonstrate mtDNA polyphyly, the mtDNA tree does not indicate that a single species exists with extensive nuclear genetic hybrids, since almost no elephants of mixed forest-savanna nuclear genotypes or intermediate morphology have been detected in savanna locales. The dearth of mixed nuclear genotypes and of intermediate morphotypes in savanna locales where forest-typical mtDNA predominates indicates that a single-species model cannot be inferred from the mtDNA pattern. Instead, only a two-species model in which male elephants are reproductively unsuccessful (Fig. 1B) can account for monophyletic nuclear gene trees in the face of polyphyletic mtDNA trees (Roca et al., 2005; Roca and O'Brien, 2005). The lack of male hybrid reproductive success would allow forest elephant-typical nuclear alleles, including those affecting morphology, to be replaced by alleles typical of savanna elephants (Fig. 1B). Since savanna males can replace nuclear genes but cannot replace the forest-derived mtDNA, the latter remains residual in the herds.

Debruyne (2005) asserts that the major argument for the two-species model presented in Roca et al. (2001) was the magnitude of DNA sequence variation, and not evidence for the completion of speciation. This is inaccurate, as the conclusions of Roca et al. (2001) were also based on the observation that common nuclear DNA haplotypes present within one species across the locales surveyed were nonetheless completely absent from the other species. For example, the haplotype *GBA-III* was carried in 96% of the chromosomes examined in savanna elephants south, east and north of the tropical forest, yet was completely absent from elephants in tropical forest locales, indicative of gene flow across the savannas but not from savanna elephants to forest elephants. Furthermore, two indels, present in genes *CHRNA1* and *VIM,* were common in forest elephants from Lope to Dzanga Sangha to the mixed population of Garamba, indicative of gene flow across the Congolian forest, but were nonetheless completely absent from savanna elephants. These patterns indicate a lack of nuclear gene flow between the two species even in the face of hybridization.

Debruyne (2005) treats the mtDNA tree as a species tree, stating that since "haplotypes are shared by both savannah and forest elephants [this] violates the assumption that they might belong to exclusive taxonomic units." Although Debruyne (2005) refers to savanna elephants carrying forest mtDNA as "hybrids," the residual mtDNA genome that savanna males cannot replace (Fig. 1B) could potentially represent less than 0.001% of the combined genetic material carried by the nuclear and mitochondrial genomes (Fig. 5D). Thus potentially >99.999% of the genetic material in one of these elephants could be derived from its savanna elephant ancestors (Figs. 1B and 5D). The polyphyletic mtDNA tree would thus be completely unrepresentative of the overall genetic status of African elephants, negating the conclusion of Debruyne (2005) that the mtDNA results can be generalized as indicating "a protracted gene flow between the two forms wherever their ranges intersect" for any locus but the single mtDNA locus itself. One can also reject the assertion of Debruyne (2005) that "Nyakaana et al. (2002) showed that the mtDNA structuring at the continental scale… contradicts the clear-cut pattern of nucDNA obtained by Roca et al. (2001) and Comstock et al. (2002)." Put simply, the monophyletic nuclear DNA pattern was obtained in the latter two studies for elephants that proved to have an mtDNA phylogeographic pattern similar to that reported by Nyakaana et al. (2002), and no contradiction of results may be inferred from comparing the nuclear gene patterns of two studies to the mtDNA patterns of a third study, when the mtDNA patterns of the elephants are actually similar for all of the studies. Finally, while Debruyne (2005) includes an erudite discussion of species concepts and evolutionary significant units, these are unfortunately applied to a gene tree inferred from a singularly unrepresentative and residual locus (mtDNA) rather than to the actual phylogeny and population structure of Africa's elephants, as revealed by multiple datasets of nuclear and morphological characteristics (Figs. 4 and 5D).

West African elephants. We did not specifically consider elephants from west of Cameroon in this analysis because they were not part of the nuclear gene studies of Roca et al. (2001, 2005), and since the collection of skulls in museums appears to be unrepresentative of the true distribution of elephants in West Africa (Groves, 2000). While determining the genetic status of current West African populations is of utmost importance for establishing their priority in conservation (Eggert et al., 2002; Blanc et al., 2003), it would nonetheless be unwise to delay conservation decisions for the rest of Africa based on lack of data for a region that contains less than 2% of the continent's remaining elephants. Most importantly, the region is far from ideal for examining natural genetic patterns to determine the systematics of *Loxodonta*. Genetic studies seek to examine natural patterns in African elephants, and to distinguish natural patterns from those influenced by human activity. Yet West Africa is a region where natural patterns have been very heavily disrupted by anthropogenic effects. Habitats in West Africa are extremely fragmented, and elephants currently inhabit only 7% of their former range in the region (Blanc et al., 2003). Widespread deforestation and hunting for the ivory trade likely disrupted natural genetic patterns to a much greater degree than elsewhere, since little of the original tropical forest remains intact, and the ivory trade caused elephant numbers to collapse in West Africa early in the 20th century (Blanc et al., 2003), much earlier and to a greater degree than in most of the continent. Large savanna male tuskers would have been selectively impacted by the ivory trade. Anthropogenic deforestation may have greatly increased opportunities for hybridization in West Africa, while isolated habitat patches may reflect the genetics of a small number of elephants that managed to locally survive poaching and habitat destruction, potentially including "hybrid swarms" (groups of genetically mixed survivors).

Thus contrary to the assertion of Debruyne (2005) that West Africa is of major importance for determining the overall systematics of *Loxondonta*, the very highly disrupted habitats of West Africa would appear to be less than ideal for examining natural habitat preferences or patterns of interaction in forest and savanna elephants.

5. Conclusions

We compared eight different datasets generated on a continent-wide level (though some did not have elephants from the far western region of Africa). Exact tests comparing forest elephant-typical versus savanna elephant-typical characteristics among African elephants revealed that datasets with similar patterns cluster into two distinct groups (Fig. 4). Those containing analyses of nuclear genes all revealed a deep and almost complete separation between forest and savanna elephants, with few or no hybrids outside of the habitat contact zone where the species meet (Roca et al., 2001; Comstock et al., 2002; Roca et al., 2005). Similar results were found for datasets analyzing the morphology of African elephants (Groves and Grubb, 2000; Debruyne, 2005). Few or no elephants with forest or intermediate morphology were detected outside of the tropical forests or the contact zone between forest and savanna habitats, a pattern supported by the dataset of Debruyne (2005), in which forest or intermediate morphology was not detected among elephants living in savanna habitats, even among those that carried a forest-typical mtDNA haplotype.

Mitochondrial DNA showed a different pattern from nuclear or morphological markers. We demonstrated using exact tests (Fig. 4) that the proportion of forest- and savanna- typical mtDNA haplotypes was similar between elephants examined by Debruyne (2005) and those examined by Roca et al. (2005). However, we also demonstrated that the mtDNA datasets displayed quite a different pattern from those present in surveys of nuclear genes, whether slow- or fast-evolving, and whether biparentally or only male-transmitted, or from those of surveys involving morphology (Fig. 4). Despite the relatively small number of savanna elephants examined ($n = 28$), the mtDNA data of Debruyne (2005) also displays a significantly ($p < 10^{-5}$) different pattern of forest- vs. savanna-typical characteristics from the morphological data of Debruyne (2005) for the same individuals.

The presence of mixed nuclear genotypes does indicate that hybridization occurs between forest and savanna elephants, yet hybrids and morphologically intermediate elephants are found in or near zones of mixed forest and savanna vegetation. The widespread distribution of forest mtDNA haplotypes in geographic regions quite distant from current tropical forest habitat suggests that hybridization in the past occurred along a shifting habitat boundary, likely increasing following periods of habitat change. The dearth of forest nuclear alleles or morphologically intermediate features in elephants in savanna habitats distant from the current forest, even among savanna elephants and populations that carry forest-typical mtDNA, suggests that there has been selection against hybrids, and specifically, given the persistence of forest-typical mtDNA in some savanna elephants, selection against the male forest-savanna hybrid elephants.

Thus, through mechanism(s) of selection against hybrids, the genetic integrity of the two parent species has been maintained, since residual forest elephant mtDNA is carried by elephants that, to the degree detectable, are completely savanna elephant-like in nuclear DNA genotype and in morphology. In his definition of the biological species concept, Ernst Mayr noted that hybrid zones could exist between validly defined species as long as the genetic integrity of the parent species remained intact (Mayr, 1969). The combined datasets of mtDNA haplotypes, nuclear DNA genotypes and morphology indicate that the genetic separation between forest and savanna elephants is overwhelmingly kept intact by selection against their hybrid male offspring. If, as Wu states, ''speciation is the stage where the populations will not lose their divergence upon contact'' (Wu, 2001) then the savanna elephant, *Loxodonta africana*, and the forest elephant, *L. cyclotis*, despite being separated by a hybrid zone, overwhelmingly maintain their genetic integrity and form distinct African elephant species.

Acknowledgments

We thank the colleagues, agencies and governments that provided samples or assistance in our primary work. We thank R. Ruggiero and the US Fish and Wildlife Service African Elephant Conservation Fund. This publication has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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